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1 Introduction

This study combines the TGFbeta neutralizing antibody, Fresolimumab, with Radiation Therapy (RT) to treat advanced metastatic breast cancer. Since this drug has been discontunued, with DOD approval, in the near future we will be switching to the small molecule TGF-beta inhibitor, Galunisertib. This report deals with Fresolimumab, which was given 5 times as either 1 mg/kg or 10 mg/kg 3-weekly schedules. RT is limited to IGRT (3 x 7.5 Gy) to 1-2 lesions with other lesions being designated as sentinels to determine abscopal responses that are hypothesized to be due to RT-induced vaccination. RT was started 10 days after the first and 3rd dose of Fresolimumab

The primary objectives are to assess safety, feasibility, and abscopal tumor regression and to monitor immune responses in these patients. The abscopal responses are assessed by imaging. The UCLA component is 3 fold: 1) to enroll patients into the clinical trial, 2) to assess immune responses using blood samples before, during and after treatment by multi-channel flow cytometry for immune monitoring, 3) to examine the effects of targeting TGF-beta on the activities and numbers of breast cancer stem cells with and without irradiation.

2 Body

2.1 Immune-monitoring

Twenty-two patients have been enrolled and the immune monitoring has been performed for all. Statistical analysis is still proceeding, but the conclusions presented here are reasonably firm.

Eleven of the patients were HLA-A2.1 positive and were assessed for responses to the tumor-specific antigen survivin before, during, and after Fresolumimab and RT. Furthermore, six of these were also examined for responses to 3 additional tumor-associated antigens. Four of the 11 patients had pre-existing high levels of CD8+ T cells to the survivin epitope. Three of these responded with increased levels after Fresolimumab plus RT. In one patient, a strong pre-treatment level decreased with time. Of the 8 who were negative before treatment, only one responded to tratment and that was initiated only on week 5.

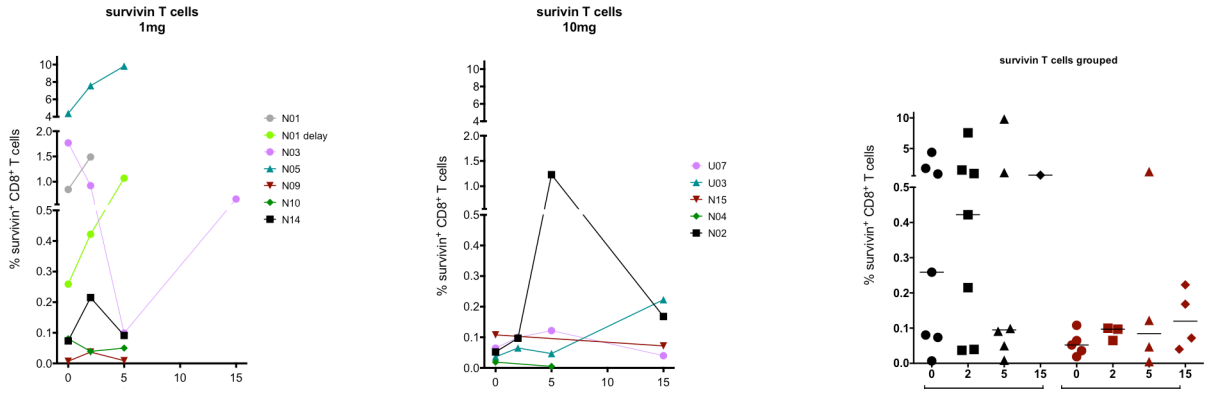


Figure 1: CD8+ survivin-specific T cells levels with time after initiation of treatment. Individual NYU patients (left) and median values for individual patients (right).

As outlined in our last report, we have moved from the use of tetramers to dextramers (Immudex USA, LLC) for their superior resolution and higher reproducibility in detecting antigen-specific responses. We have compared survivin responses with those to other antigens seen by patients with breast cancer. The aim was to detect if there was epitope spreading, which we have shown is important for tumor regression. The theory is that this may be required for clinical activity and that RT may promote this effect in the presence of anti-TGFbeta. All HLA-2.1+ patients that had previously been tested for survivin responses were included provided there was material available. All in all, 7 old and 3 new patients were tested for circulating T cells reactive against Mucin 1 (Muc 1), Her-2/neu and JARID1B (Figures 2 and 3). This choice of tumor-associated antigens was based on the literature and on previously measured antibody responses in our patient pool (see previous report). An initial screen for survivin-reactive tumor-specific T cells The numbers of samples are small (5) but although many responses were minimal (Figures 2 and 3), there was some “trackin for Her2 and Muc1, but none for Jarid1B.

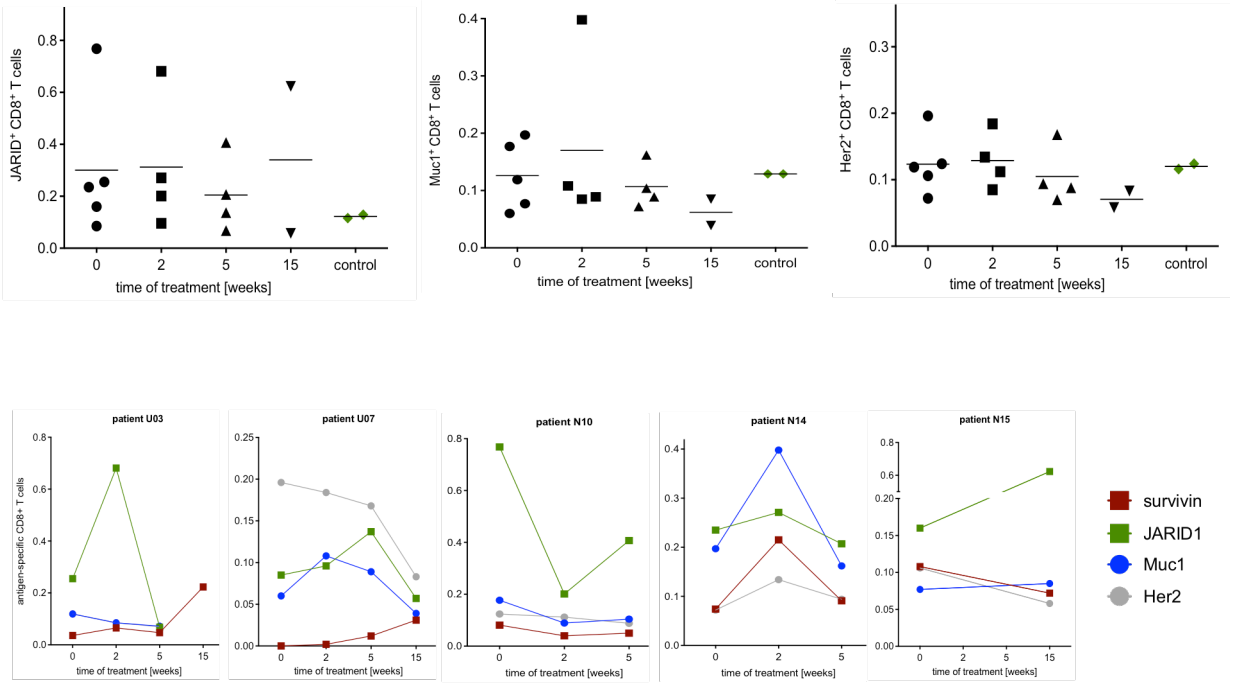


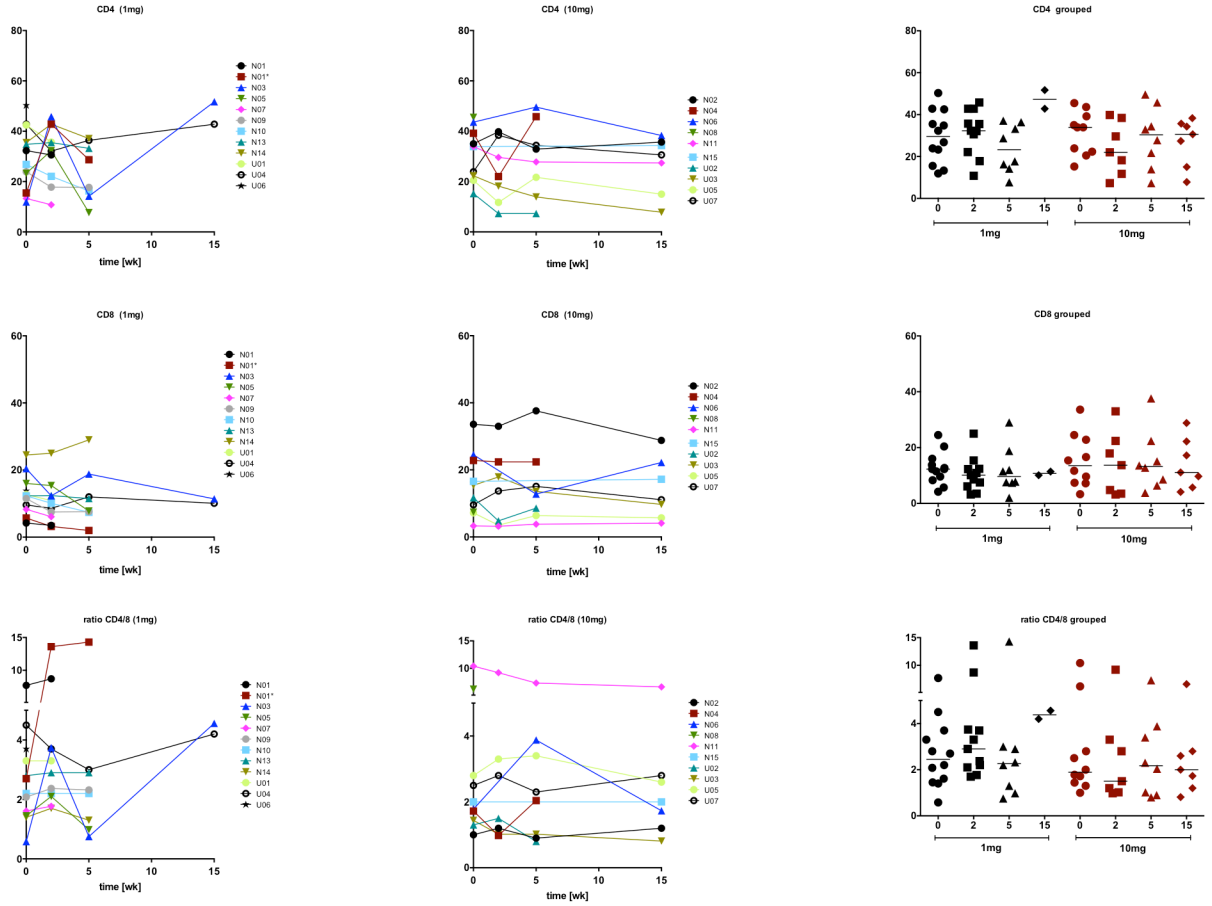
Figure 2: Dextramer testing for JARID1B+, Muc1+ and Her2+ specific T cells (top) and individual PBMC reactivity to tumor-associated antigens (bottom).

2.2 Monitoring the patient’s general immune status

As outlined in our last report, we have extended our lymphoid and myeloid staining panels with the following markers: PD-1, CD3, CD45RA, CCR7 and CD20, CD56 and CD123. This has allowed us to look at naïve, effector and memory subsets as well as NK cells, B cells and plasmacytoid DCs in addition to what was included previously. The vast amount of data that has emerged is of great interest and is still being statistically analysed, but there are changes with tretment that are of interest. Five out of 18 patients showed an increase in CD4+ cell mobilization on week 2 that had

reverted to normal by week 5. For 3 of these the increase was associated with a decrease in CD8+ cells. h17 cells generally decreased after Fresolumimab with a

Figure 3: CD4 and CD8 cell numbers and ratios before, during, and after treatment.



resultant large change in Th1:Th17 ratios.

One third of the 18 patients had pre-exisitng myelopoiesis as indicated by high circulating myeloid cell numbers. Noticibly, the fraction of myeloid cells within the circulating PBMC pool appears to increase during the initial phase of treatment, as does the subset of intermediate monocytes within that myeloid population which we were able to appreciate after optimizing our monocyte gating strategy. We have shown in animal models that myelopiesis has a profound effect on lymphocyte responses and bone marrow mobilization. Remarkably, all these patients showed an increase in T regulatory cells 2 weeks after the start of treatment (Figure 4). Two patients out of 7, for whom samples were available for the whole treatment course, showed dramatic late increases in Tregs. We have also shown increases in B cells and mature dendritic cells and currently we are assessing the correlations between these responses.

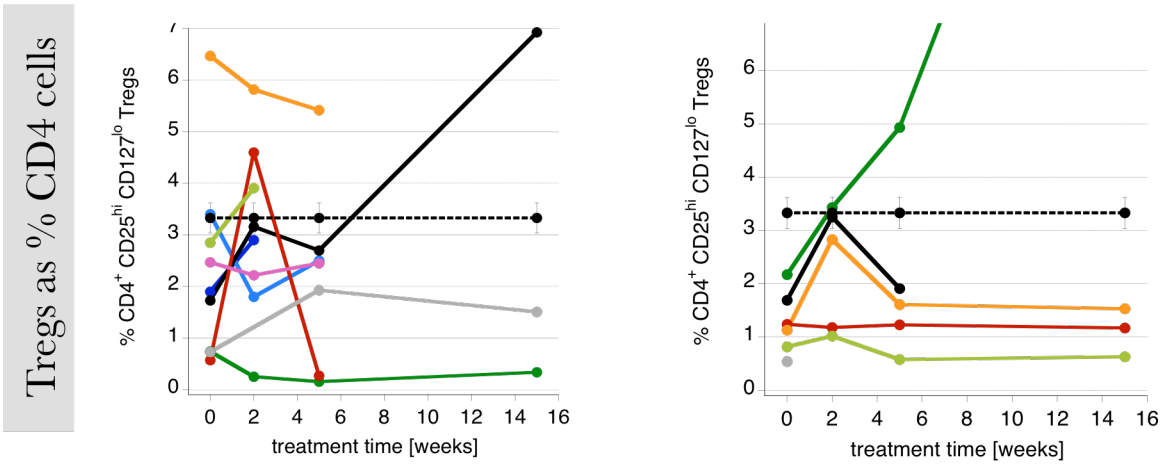


Figure 4: Treg cells as a % of CD4+ cells. Similar data were obtained for % of all lymphocytes.

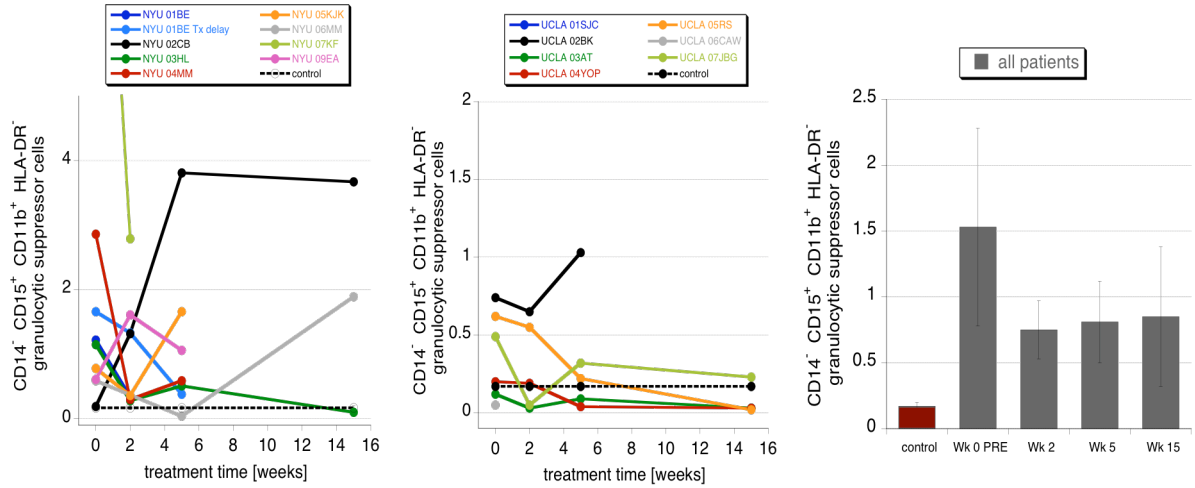


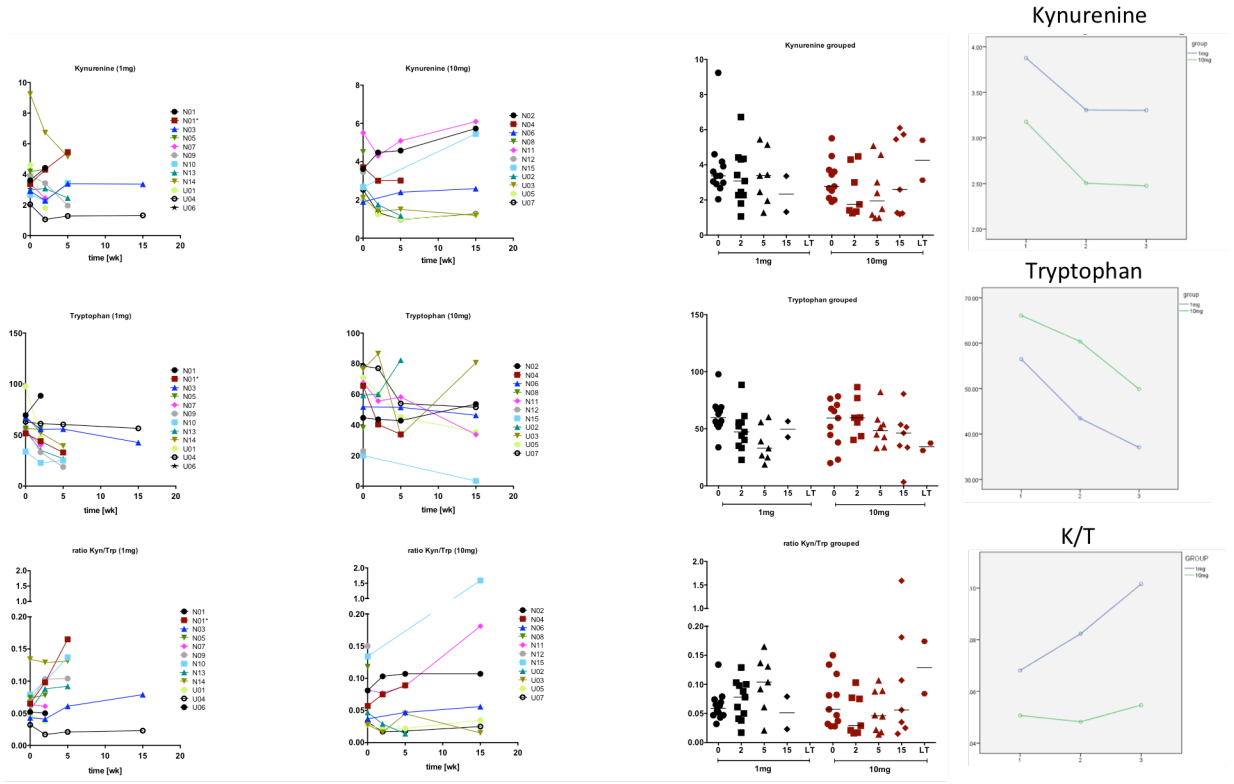
Figure 5: Cells of the myeloid suppressor cell lineage before and after treatment.

Myeloid cell subsets were evaluated in depth. The most interesting finding was that cells of the myeloid-derived lineage that are generally accepted to be immunosuppressive, were high in about 70% of patients and almost universally fell after Fresolumimab treatment, suggesting that TGF-beta may be responsible foir maintaining this subset (Figure 5). One patient, however gave an opposite result with dramatic increases with time, starting from a low “normal” level.

We have been interested in the levels of kynurenione and tryptophan during treatment. Indoleamine 2,3-dioxygenase (IDO) catalyzes the rate-limiting step of tryptophan (Trp) degradation along the kynurenine (Kyn) pathway. IDO is considered to be a fundamental immune escape mechanism for tumor cells and increasing ratios of kynurenine to tryptophan have been associated with disease. We noted marked decreases in both kynurenine and tryptophan that were statistically significant and appeared to be related to dose of Fresolimumab (Figure 6). This is of particular interest

because patients receiving the high 10mg dose survived 6 months longer than those getting the lower 1mg dose.

Figure 6: Changes in kynurenine and tryptophan after Fresolimumab and RT treatment.



2.2 Effects of TGF-beta on breast cancer stem-cells

Recent preclinical and clinical data support that solid cancers including breast cancers are organized hierarchically with a small population of cancer stem cells (CSCs), capable of re-growing the entire tumor while their progeny lack this ability. We and others reported that breast CSCs (BCSCs) are relatively resistant to ionizing radiation and after irradiation, the surviving BCSCs are recruited from a quiescent state (G0) into an active cell cycle, allowing repopulation of the tumor. Furthermore, we have shown that RT can induce reprogramming of non-tumorigenic cancer cells to generate new cancer stem cells (induced cancer stem cells, iCSCs). The mechanisms involved require the re-expression of the stem cell transcription factors Oct-4, Sox2 and Nanog. Interestingly, this re-expression was higher in polyploid cells.

TGFβ activation is a regulator of BCSCS expansion and the project aims at investigating its effect on reprogramming of non-BCSCS into BCSCs. Interestingly, we have observed varying effects for BCSCs when breast cancer cells were treated with an inhibitor of the TGFβ receptor. Several schedules have been investigated using 3 different cell lines. TGF-β seems to inhibit reprogramming of SUM159PT, a claudin-low cell line, while it enhances reprogramming in MCF7, a luminal cell line. MDA-MB-231, a

basal cell line, was very sensitive to TGF-βR inhibition resulting in significant toxicity. These divergent data that may depend upon the origin of the breast cancer are important if we are to further understand the effects of Fresolimumab and RT on BCSCs and this work is ongoing.

3. Problems Encountered

There have been no problems with subject enrollment or retention. We have received all subjects from the UCLA Department of Hematology-Oncology as referrals. There are no problems to report regarding the conduct of study procedures, consenting, confidentiality or anything else that would be considered reportable. All SAEs and AEs have been reported to NYU and the UCLA IRB as per protocol.

Unfortunately Fresolimumab was removed from availability by the manufacturer, but the use of a small molecule TGF-beta inhibitor has now been approved. Dr. Formenti visited UCLA early in the year and met with our clinical team, including Dr. Glaspy, the Director of the JCCC Clinical Research Unit and of the JCCC Women's Cancer Research Program, and Dr. Kupelian, Vice Chair for Clinical Studies in Radiation Oncology, both of whom agreed to the trial. We will continue the trial with a small molecule inhibitor, Galunisertib, having recruited two additional breast cancer clinicians and an additional research nurse. IRB approval has been given and we expect to start the trial any time now.

4. Future Directions

The enrollment for Fresolimumab is complete as is the immune monitoring. We are data mining, summarizing, and statistically analyzing data. We expect to be completed this soon and have a publication ready within the next 2 months.

The anti-TGFbeta studies with cancer stem cells will and the importance of this pathway in radiation-induced reprogramming is essentially complete and has resulted in numerous publications.

5. Key Research Accomplishments

Overall

- Trial recruitment for Fresolimumab is complete: Galunisertib about to start
- Immune monitoring of existing patient samples is complete and is undergoing statistical analysis
- We have developed a new assay for kynurenine and tryptophan to assess IDO activity that is providing useful data
- TGF-beta affects non-stem cell reprogramming by radiation exposure in a manner that depends on the origin of the breast cancer cell line.

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